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14. ABSTRACT

Rsu1 and PINCH1 are required for migration of MCF10A mammary epithelial cells. Depletion of Rsu1 or PINCH1 by siRNA alters the distribution and localization of the FA proteins vinculin, paxillin and talin. While control cells display highly organized peripheral staining of FA proteins, Rsu1 or PINCH1 knockdown cells exhibit disorganized staining throughout the cell body. However, the level of the FA proteins in the cells did not change in response to Rsu1 or PINCH1 knockdown. Cells depleted of Rsu1 or PINCH1 adhere poorly and have disorganized caveoli but no loss of caveolin, β1 or β3 integrin or EGF-R. Examination of actin cytoarchitecture by confocal microscopy showed the loss of stress fibers and the formation of actin rich structures at the cell periphery in Rsu1- or PINCH1-depleted cells. While migration is inhibited in the Rsu1 and PINCH1 depleted cells Rac1 can be activated by EGF, the modulator of migration in these cells. These results demonstrate a critical role for Rsu1 and the IPP complex in proper formation of FA sites and the actin cytoskeleton as well as in cell adhesion and migration.

15. SUBJECT TERMS
Breast, Mammary, IPP complex, Rsu1, ILK, Parvin, Ras, GTPases, actin cytoskeleton, Focal Adhesion

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Introduction

Cell migration is an essential cellular process and deregulation of this process may result in serious consequences such as tumor metastasis[1,2]. Numerous studies have demonstrated that the IPP complex is linked to cell adhesion and cell migration[3,4]. Our lab has found that Rsu-1 binds to the IPP complex through PINCH1[5] and the absence of either Rsu-1 or PINCH1 proteins results in a change in epithelial cell migration[3,6,7]. In addition, the levels, the forms and the subcellular localization of Rsu-1 and IPP proteins are altered in breast cancer cells compared to non-tumorigenic mammary epithelial cells[3]. In particular, the Rsu-1 variants contribute to breast cancer cell migration. **Therefore, it is my hypothesis that Rsu-1 splice variant(s) contribute to cell migration by functioning as a regulator of essential migration processes. In addition, the presence of Rsu-1 splice variant(s) may enhance migration in tumorigenic mammary epithelial cell lines.** Since focal adhesion formation and stability, G-protein signaling and actin polymerization are fundamental in the process of cell migration[1,7,8] ,the information that we gather relevant to these processes will further our understanding of the role of the IPP complex and Rsu-1 in the regulation of cell migration. This research is proposed to elucidate the mechanistic role of Rsu-1 in breast cancer cell migration.

Body

Research Plan

Phase 1: Identify the Rsu-1 splice variant(s) that control cell migration. (Year 1).

Task 1. Identify the existence of Rsu-1 splice variant(s) in non tumorigenic (MCF-10A, MCF-7) and tumorigenic (MDA-MB-231) mammary epithelial cell lines. (Months 1-3)

1a. RT-PCR and Western Blot analysis will be used to determine mRNA and protein expression levels of Rsu-1 variant(s).

Western Blot analysis had been performed on non tumorigenic (MCF-10A, MCF-7) and tumorigenic (MDA-MB-231, MDA-MB-468) mammary epithelial cell lines to assess protein expression levels of Rsu-1 variant(s). The results in figure 1 demonstrate that the ER-, highly tumorigenic human breast cancer cell lines (MDA-MB-231 and MDA-MB-468) express both the p33 and p29 kDa isoforms while the ER+ cell lines express only the p33 isoform.

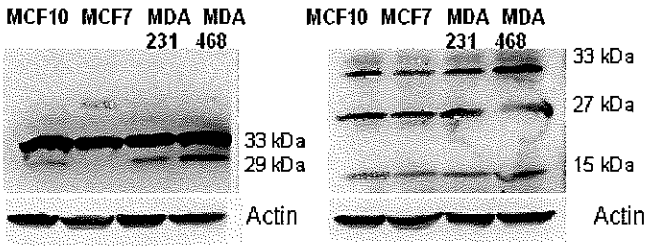


Figure 1. Equal amounts of tumor cell line lysates (100 µg) were used for western blotting with antibodies to N-terminal and COOH terminal regions of Rsu-1. Detection of actin was used as a loading control.

1b. The RT-PCR products will be sequenced to confirm their identity as Rsu-1 altered splice product(s) and to categorize their exon structure.

RNA was isolated from non tumorigenic (MCF-10A, MCF-7) and tumorigenic (MDA-MB-231, MDA-MB-468) mammary epithelial cell lines and used for reverse transcription and amplification with primers for the 5' and 3' UTR regions of the cDNA. The products were separated on agarose gels and the Rsu-1 specificity detected by southern blotting with Rsu-1 specific probe. The cDNA products were cloned into plasmid vectors and sequenced. (Figure 2)

The sequencing revealed that the cell line expressing the p29 product also expressed an exon 8-deleted form of the Rsu-1 RNA.

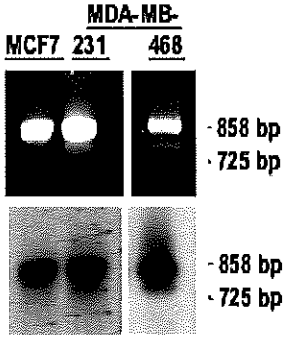


Figure 2. The RT-PCR of total RNA from non tumorigenic (MCF-7) and tumorigenic (MDA-MB-231, MDA-MB-468) mammary epithelial cells lines was performed. In addition, the RT-PCR products were analyzed by southern blot with an Rsu-1 specific probe, TA-cloned and sequenced.

Task 2. Depletion strategy to determine the impact of each variant found in breast cancer cells on cell migration (Months 3-6)

2a. Use our tested Rsu-1 siRNA to deplete the expression of the full length Rsu-1 transcript, the p29 Rsu-1 splice variant and other variants in non tumorigenic and tumorigenic mammary cell lines.

Studies have been initiated using the MCF-10A cell line based on its ability to mimic a normal breast epithelial environment. The siRNA used on these studies successfully depleted the expression of all the Rsu-1 RNAs as seen below (Figure 3).

2b. RT-PCR and Western Blot analysis will be use to measure the efficacy of the Knockdown

The efficacy of the Rsu-1 knockdown was measured by Western Blot analysis. Western Blot revealed that depletion of Rsu-1 causes significant reduction in PINCH1, but PINCH1 depletion results in only a modest reduction in Rsu-1. This implies a function for Rsu-1 in regulating the IPP complex through PINCH1.

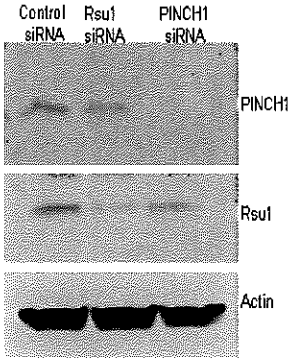


Figure 3. Cells were transfected with siRNA and at 96 hrs post transfection cells were lysed. Equal amounts of MCF-10A cell line lysates (80 µg) were used for western blotting with antibodies to N-terminal region of Rsu-1 and PINCH1. Detection of actin was used as a loading control.

2c. Cell migration capacity will be evaluated by the use of techniques that include the wound healing, assay by scarring and Oris plate migration assay, transwell migration assay and live-cell imaging studies.

Cell migration capacity was evaluated by the use of the Oris plate migration assay. MCF-10A cells were transfected with a negative control, Rsu-1 or PINCH1 siRNA and plated in Oris^R migration plates. The wells contain stoppers that cover a field into which cells can migrate. Following removal of the stoppers migration was documented and quantified. Quantitation was performed by staining cells and reading absorbance within field of migration. Western blot analysis was performed to confirm Rsu-1 and PINCH1 knockdown. Depletion of the full length Rsu-1 and PINCH1 correlates with decreased cell migration. The results indicated that the depletion of Rsu-1 or PINCH1 inhibited MCF-10A cell migration in response to EGF (Figure 4).

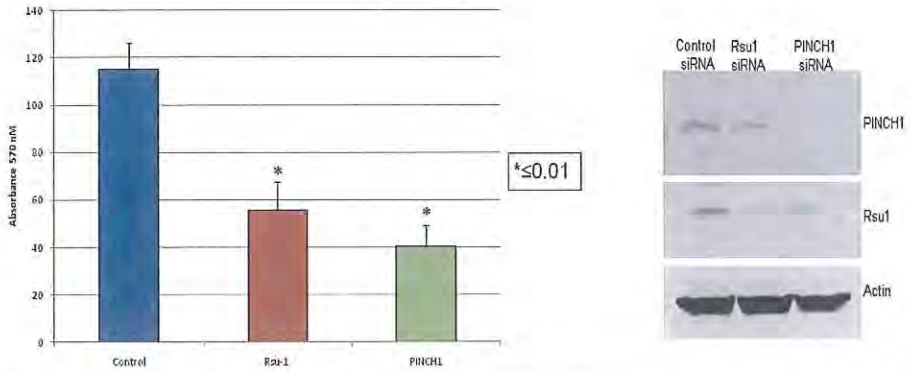


Figure 4. MCF-10A cells were transfected with negative control, Rsu-1 and PINCH1 siRNA and plated in migration plates (Oris). At 72 hrs post transfection the migration assay was initiated (T=0). Cells were photographed at T=0 and T=24 hr then fixed, stained with crystal violet and quantitated (570 nM). Graphic representations of results from 5 stained wells (Mean+SE). Cell lysates were collected at T=96 hrs for western blot analysis.

Task 3. Expression of the different splice variant(s) will also be utilized to complete our studies. (Months 6-12)

3a. Use of siRNA directed at the 3'UTR of the endogenous Rsu-1 transcript to deplete cells of endogenous Rsu-1 RNA and protein in the presence of vector-encoded Rsu-1 variants.

Knockdown of Rsu-1 by siRNA directed to the 3' UTR (targets human Rsu-1 sequence) has been done successfully. The known Rsu-1 variants have been epitope tagged (Myc). The myc Rsu-1 is encoded by the mouse Rsu-1 sequence and is not a target of the siRNA. The full length Rsu-1 sequence has been incorporated into a lentivirus system. I was able to restore the expression of Rsu-1 on Rsu-1 depleted MCF-10A cells (Figure 5). Since the Rsu-1 splice variants have been Myc tagged, the next step will be to incorporate the independent splice variants into the lentivirus system beginning with the 29 kDa variant. There is still some work in progress to maximize the levels of Rsu-1 re-expression by lentiviral infection.

Figure 5. MCF-10A cells were transfected with Control and Rsu-1 siRNA's. Lentivirus containing the full length Rsu-1 sequence was added to the cells. Cell lysates were collected at T=48 hrs post lentiviral infection. Equal amounts of lysates (80 µg) were used for western blotting with antibodies to the N-terminal region of Rsu-1. Detection of actin was used as a loading control.

3b. *The different Rsu-1 variants will be transfected/infected into MCF-10A, MCF-7 and MDA-MB-231.*

These studies have been initiated with the MCF-10A cells.

3c. *Cell migration capacity will be evaluated by the use of techniques that include the wound healing assay by scarring and Oris plate migration assay, transwell migration assay and live-cell imaging studies.*

These studies have been initiated with the MCF-10A cells.
The outcomes from Phase 1 of the project will identify the Rsu-1 splice variant(s) that regulate cell migration in non tumorigenic (MCF-10A, MCF-7) and tumorigenic (MDA-MB-231) mammary epithelial cell lines.

Phase 2: Determine the mechanism by which Rsu-1 variant expression regulates intracellular processes that control cell migration (Year 2)

Task 1. Explore the effect of the depletion of the full length Rsu-1 transcript, Rsu-1 splice variant(s) and the members of the IPP complex in MCF-10A, MCF-7 and MDA-MB-231 cells in the context of cell migration. (Months 1-2)

1a Use of siRNA directed at the different members of the IPP complex in combination with siRNA's directed to the p33 Rsu-1 and p29 Rsu-1

These studies have not yet been initiated with the MCF-10A cells.

1b. The efficacy of the knockdowns will be quantified by Western Blot and Immunofluorescence

Western Blot Analysis data showed in Phase 1 Task 2b. The efficacy of the Rsu-1 and PINCH1 knockdown had been also measured by confocal microscopy (Figure 6).

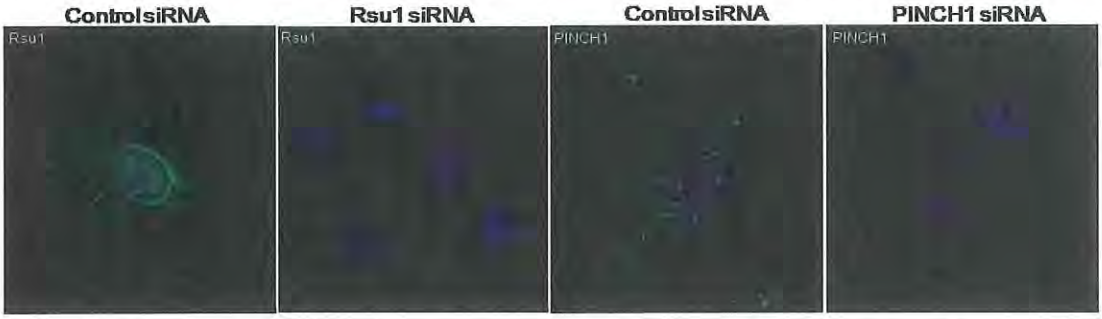


Figure 6. MCF-10A cells were transfected with Control, Rsu1 and PINCH1 siRNA and plated on coverslips. Cells were fixed at 96hrs and assayed by immunofluorescence using Rsu-1 and PINCH1 antibodies. Nuclei were counterstained with DAPI

1c. Determine the composition of focal adhesions (vinculin, ILK, α-actinin, FAK, P-FAK) by immunofluorescence using confocal microscopy. We will look for the effect of focal adhesion number, size, concentration and composition by double staining.

Confocal microscopy and western blot analysis had been performed to analyze the distribution, localization and the expression of FA proteins such as: vinculin, paxillin and talin. Depletion of Rsu-1 or PINCH1 by siRNA in MCF10A cells resulted in a change in the distribution and localization of the FA proteins vinculin, paxillin and talin. Control cells showed a peripheral and highly organized staining of FA proteins while the knockdown cells exhibit disorganized staining throughout the cell body. However, western blotting revealed that the level of the FA proteins did not change in response to Rsu-1 or PINCH1 knockdown.

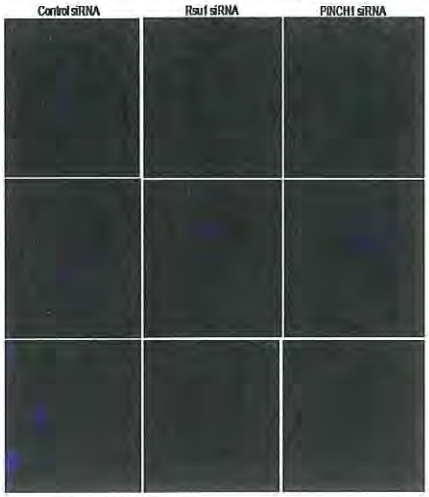
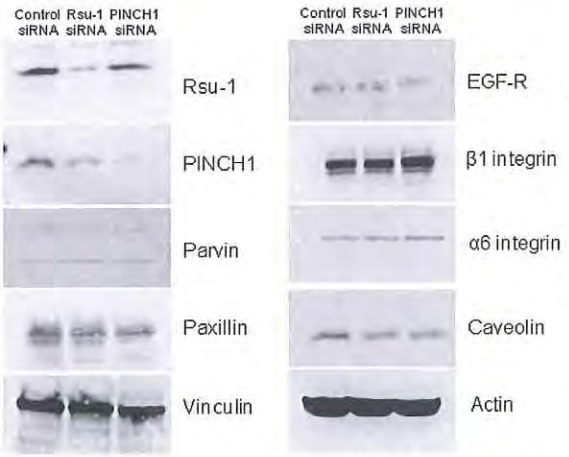
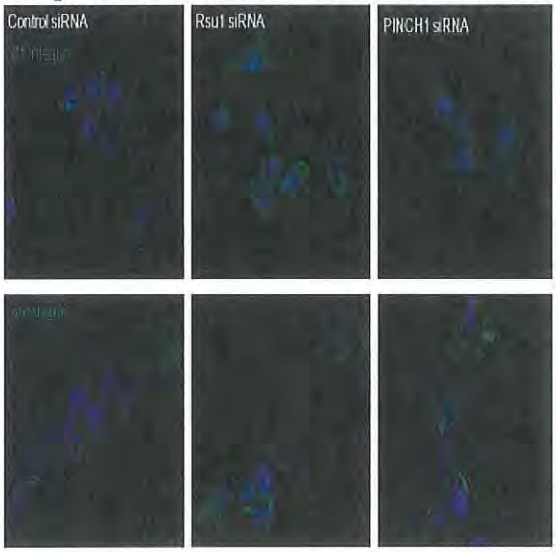


Figure 7. MCF-10A cells were transfected with Control, Rsu1 and PINCH1 siRNA and (A) plated on coverslips. Cells were fixed at 96hrs and assayed by immunofluorescence using Paxillin, Vinculin, Talin, Rsu-1 and PINCH1 antibodies. Nuclei were counterstained with DAPI. (B) Lysates were collected 96 hrs post transfection and analyzed by western blot.



Integrin signaling and recycling is required for FA site formation, adhesion and migration. The effect of Rsu-1 and PINCH1 depletion on the distribution, localization and expression of integrins that are commonly associated with cell migration was examined by confocal microscopy and western blot analysis. In addition, caveolin membrane protein was analyzed because of its pivotal role in receptor independent endocytosis, integrin recycling and its association with the RIPP complex. MCF-10A cells depleted of Rsu-1 or PINCH1 adhere poorly to substrate and have reduced and disorganized caveolae as well as β1 and α6 integrin complexes (Figure 8). However, western blotting revealed that the level of the FA proteins did not change in response to Rsu-1 or PINCH1 knockdown.



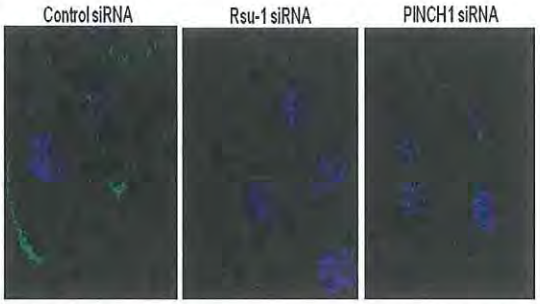


Figure 8. MCF-10A cells were transfected with Control, Rsu-1 and PINCH1 siRNA and plated in coverslips. Cells were fixed at 96hrs and assayed by immuno-fluorescence using TRITC-phalloidin, caveolin and integrin antibodies. Nuclei were counterstained with DAPI.

Task 2. Determine the effect of Rsu-1 and the IPP complex on actin polymerization. (Months 4-6)

2a. Use of phalloidin labeling and/or cells expressing GFP-actin as a marker.

2b. Immunofluorescence confocal microscopy will be used to assess this.

Phalloidin labeling had been used to assess changes on actin polymerization in Rsu-1 and PINCH1 knockdown cells. Rsu-1 and PINCH1 depletion resulted in a loss of stress fibers and disorganized actin cytoskeleton staining (Figure 9). A reorganization of the actin cytoskeleton correlated with loss of FAs in Rsu-1 and PINCH1 depleted cells.

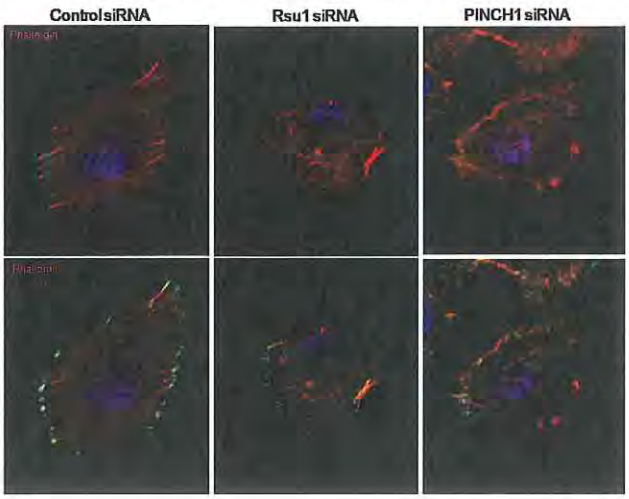


Figure 9. MCF-10A cells were transfected with Control, Rsu-1 and PINCH1 siRNA and plated on coverslips. Cells were fixed at 96hrs post-transfection and assayed by immunofluorescence using TRITC phalloidin. Cells were also costained with TRITC phalloidin and viinculin antibodies. Nuclei were counterstained with DAPI.

Task 3. Determine the effect of Rsu-1 and the IPP complex on different GTPases required for cell migration including Rac, CDC42, and Rho GTPases. (Months 6-10)

3a. Analyze levels of Rac-GTP and CDC42-GTP by pulldown assays

The Rsu-1- or PINCH1-depleted cells retain the ability to activate Rac and cdc42 in response to EGF stimulation. Rho-GTPase studies are underway.

3b. Transfect plasmid for FRET probes that will allow the measurement of Rho GTPases activity in individual cells. Include transfection of Rho where necessary to generate a significant signal.

These studies have not yet been initiated.

Task 4. Differentiate effects of Rsu-1 variant expression on focal adhesion formation resulting in guanine nucleotide exchange factor, α-PIX, activation leading to Rac-GTP versus JNK activation leading to Rac-GTP activation. (Months 10-12)

4a. Use of JNK inhibitors in conjunction with Rsu-1 variant and look for effect on variant controlled migration

These studies have not yet been initiated.

4b. Use knockdowns of PIX family proteins in conjunction with Rsu-1 variant

These studies have not yet been initiated.

4c. Measure Rsu-1 variant induced migration and Rac/cdc42 activation

These studies have not yet been initiated.

4d. Measure effects of variants on JNK activation (kinase assay) and α, β-PIX

These studies have not yet been initiated.

The outcomes from Phase 2 of the project will help to define the mechanistic role of Rsu-1 and the IPP complex in the regulation of cell migration in breast cancer.

Key Research Accomplishment

- Depletion of Rsu-1 causes significant reduction in PINCH1, but PINCH1 depletion results in only a modest reduction in Rsu-1. This implies a function for Rsu-1 in regulating the IPP complex through PINCH1.
- Rsu-1 or PINCH1 depletion results in a decrease in mammary epithelial cell migration in MCF-10A cells.
- The depletion of Rsu-1 or PINCH1 by siRNA in MCF10A mammary epithelial cells results in disruption of focal adhesions and loss of cell adhesion. This occurs through altered distribution and localization of the FA proteins β1 integrin, vinculin, talin and paxillin even though the absolute levels of the FA proteins do not change. Caveoli are disrupted in Rsu1- and PINCH1-depleted cells but level of caveolin remains constant. Cells depleted of Rsu-1 or PINCH1 exhibit loss of actin stress fibers.
- The disorganized caveoli and FAs in Rsu-1 and PINCH1 knockdown cells could be explained by a defect in integrin recycling that may lead to a decrease in cell migration. Rsu-1 and PINCH1 may be playing a role in endocytic transport.

Reportable Outcomes

Oral Presentations

R.P. González-Nieves. *Regulation of Cell Migration in Mammary Epithelial Cells.* Student Seminar at Uniformed Services University of the Health Sciences (USUHS). December 2010.

Abstracts/Posters

R.P. González-Nieves, Akiko DeSantis, Mary Lou Cutler. *Rsu-1 and PINCH1 are Required for Mammary Epithelial Cell Adhesion and Migration.* Poster presentation at USUHS Research Week, May 2010. Appendix #1

R.P. González-Nieves, Akiko DeSantis, Mary Lou Cutler. *Regulation of Cell Migration in Mammary Epithelial Cells by Rsu1 and the IPP complex.* Poster presentation at Era of Hope Meeting in Orlando, Florida, August 2011. Appendix #2

Conclusion

Rsu1 and PINCH1 are required for MCF10A adhesion and migration *in vitro*. Depletion of Rsu-1 or PINCH1 by siRNA in MCF10A cells results in a change in the distribution and localization of the FA proteins vinculin, paxillin and talin. Control cells showed a peripheral and highly organized staining of FA proteins while the knockdown cells exhibit disorganized staining throughout the cell body. However, western blotting revealed that the level of the FA proteins did not change in response to Rsu-1 or PINCH1 knockdown. MCF10A cells depleted of Rsu-1 or PINCH1 adhere poorly to substrate and have reduced and disorganized caveoli but no loss of caveolin, β1 or β3 integrin or EGF-R expression. However, the Rsu-1- or PINCH1-depleted cells retain the ability to activate Rac in response to EGF stimulation. Examination of actin cytoarchitecture by confocal microscopy showed the loss of stress fibers and the formation of actin rich structures at the cell periphery in Rsu-1- or PINCH1-depleted cells. These results demonstrate a critical role for Rsu-1 and the IPP complex in proper formation of FA sites and the actin cytoskeleton as well as in cell adhesion and migration. The completion of these studies, by determining the role of the Rsu-1 splice variants on the IPP complex function, will help to define the mechanistic role of this complex in breast cancer cell migration and identify the impact of loss of full length Rsu-1 on disease progression.

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Appendices

R.P. González-Nieves, Akiko DeSantis, Mary Lou Cutler. *Rsu-1 and PINCH1 are Required for Mammary Epithelial Cell Adhesion and Migration.* Poster presentation at USUHS Research Week, May 2010.

Cell adhesion and migration require integrin activation, the formation and dissolution of focal adhesion (FAs), and linkage of actin cytoskeleton to the FAs. The RIPP (Rsu1, ILK, PINCH1, Parvin) complex regulates FA formation via binding of the adaptor protein ILK to β-integrin, PINCH1 and Parvin and the linkage of Rsu1 to the complex via PINCH1. The RIPP complex is altered in fibrotic disease and cancer thus contributing to changes in adhesion and migration. Our studies focus on the mechanism by which this occurs. The depletion of Rsu1 or PINCH1 by siRNA in MCF10A mammary epithelial cells results in altered distribution and localization of the FA proteins β1 integrin, vinculin, talin and paxillin even though the absolute level of the FA proteins do not change. Cells depleted of Rsu1 or PINCH1 adhere poorly to substrate, fail to migrate and exhibit disorganized caveoli as well as loss of actin stress fibers. The depletion of Rsu1 results in greater than 50% reduction in PINCH1, suggesting that Rsu1 may function by regulating levels of PINCH1. This hypothesis was examined in several ways. Reconstitution of Rsu1-depleted cells with mutant that fails to bind PINCH1 did not alter PINCH1 localization, indicating that binding to Rsu1 is not required for PINCH1 localization. In contrast, expression a PINCH1 mutant that does not localize to FAs blocked FA-association of Rsu1, suggesting that FA-associated PINCH1 is necessary for Rsu1 migration to these sites. These results support a critical role for Rsu1 in maintaining adequate levels of PINCH1 and for PINCH1 in localizing Rsu1 to FAs, thus enhancing FA formation and stability.

Appendix #2

R.P. González-Nieves, Akiko DeSantis, Mary Lou Cutler. *Regulation of Cell Migration in Mammary Epithelial Cells by Rsu1 and the IPP complex.* Poster presentation at Era of Hope Meeting in Orlando, Florida, August 2011.

As a component of invasion and metastasis the ability of cancer cells to migrate is a critical issue in breast cancer progression. The IPP complex functions as an adaptor between integrins and the actin cytoskeleton and contributes to the regulation of signaling pathways. The IPP focal adhesion (FA) complex is composed of the proteins **I**LK, **P**INCH1 and **P**arvin as well as an associated protein Rsu1, which binds to the LIM 5 domain of the LIM only protein PINCH1. Depletion of the Rsu1 protein or other members of the IPP complex inhibits mammary epithelial cell migration. ILK and PINCHI are elevated in breast cancer cells compared to non-tumorigenic mammary epithelial cells. Rsu1 alternative splice variants that fail to bind PINCH1 are detected in breast cancer cell lines and their presence correlates with an increase in cell migration and Rac activation. Realtime PCR analysis of Rsu1 RNA from primary human breast tumors and metastases identified lymph node metastases that expressed only splice variant versions of Rsu1, emphasizing the importance of these variants in the progression of the disease.

Depletion of Rsu1 or PINCH1 by siRNA in MCF10A cells results in a change in the distribution and localization of the FA proteins vinculin and paxillin. Control cells showed a peripheral and highly organized staining of FA proteins while the knockdown cells exhibit disorganized staining throughout the cell body. However, western blotting revealed that the level of the FA proteins did not change in response to Rsu1 or PINCH1 knockdown. MCF10A cells depleted of Rsu1 or PINCH1 adhere poorly to substrate and have reduced and disorganized caveoli but no loss of caveolin, β1 or β3 integrin or EGF-R expression. However, the Rsu1- or PINCH1-depleted cells retain the ability to activate Rac in response to EGF stimulation. Western Blot and confocal microscopy revealed that depletion of Rsu1 causes significant reduction in PINCH1, but PINCH1 depletion results in only a modest reduction in Rsu1. This implies a function for Rsu1

in regulating the IPP complex through PINCH1. Examination of actin cytoarchitecture by confocal microscopy showed the loss of stress fibers and the formation of actin rich structures at the cell periphery in Rsu1- or PINCH1-depleted cells. These results demonstrate a critical role for Rsu1 and the IPP complex in proper formation of FA sites and the actin cytoskeleton as well as in cell adhesion and migration. The completion of these studies, by determining the role of the Rsu1 splice variants on the IPP complex function, will help to define the mechanistic role of this complex in breast cancer cell migration and identify the impact of loss of full length Rsu1 on disease progression.